CHEMISTRY LETTERS, pp. 1771-1774, 1993. © 1993 The Chemical Society of Japan

Effect of Surface Hydrophilicity of an Indium Oxide Electrode on Direct Electron Transfer of Myoglobins

Masato TOMINAGA, Toru KUMAGAI, Shinya TAKITA, and Isao TANIGUCHI*
Department of Applied Chemistry, Faculty of Engineering, Kumamoto University,
2-39-1, Kurokami, Kumamoto 860

Effect of hydrophilicity of the surface of an In_2O_3 electrode on the electrode reaction of myoglobins was clearly demonstrated for the first time. At the highly hydrophilic surface of an In_2O_3 electrode, well-defined redox waves of myoglobins from both horse heart and sperm whale skeletal muscle were observed with the heterogeneous electron transfer rate constants ($k^{o\prime}$) of ca. 3×10^{-4} and 1×10^{-4} cm s^{-1} at pH 6.5, respectively.

Recently, electrode reactions of metalloproteins have received active attention,[1] because functional electrodes, on which rapid electron transfer of the proteins takes place, have been developing rapidly and roles of functionalized surfaces of the electrodes become increasingly clear. Myoglobin is one of the extensively studied heme proteins,[2] having a biological function of oxygen storage and transport in its reduced form, but the redox response of this protein at an electrode is difficult to observe. Very recently, we have fortunately found[3] that a well-defined redox wave of horse heart myoglobin was observed at a clean In_2O_3 electrode for a purified sample. This is because components of higher (mainly doubly) molar masses and of denatured apomyoglobin, which are contained in commercially available samples, covered on the In_2O_3 electrode surface to inhibit the electron transfer of native myoglobin at the electrode.[3] Also, rapid electron transfer of myoglobin was observed only at a very clean In_2O_3 electrode, and the electrochemical response was not enough reproducible at an electrode after cleaning by a usual procedure.[3,4] Of course, electrode reactions are known to depend on the surface conditions of the electrode. Particularly, the surface nature affects very much on protein electrochemistry.[5,6] The hydrophilicity of the electrode surface has been considered to be one of the important factors and a subject of discussion for a long time.[7] However, no clear relationship between the surface hydrophilicity and the kinetics of an electrode reaction has so far been reported.

To make more clear the role of the surface nature on the electrode reaction of myoglobin, in the present study, the effect of the hydrophilicity of In_2O_3 electrode surface was examined quantitatively, and clear dependence of the surface hydrophilicity on the rate of electron transfer of myoglobin at an electrode was given, for the first time. Also, an interesting difference in electrochemical behavior between horse and sperm whale myoglobins was observed.

The surface tension of an In_2O_3 electrode was measured in water at 24 ± 1 °C, using a Shimadzu ST-1 surface tensometer by the well-known Wilhelmy method. For the surface tension measurements, In_2O_3 electrodes were prepared (by Kinoene Optics Corp.) by vacuum evaporating thin In_2O_3 films (ca. 30 nm) on both sides of thin (0.3 mm) glass plates (10 x 5 mm). The In_2O_3 electrode was cleaned by ultrasonication in a

1% aqueous New-Vista (the anionic surfactant, AIC Corp.) solution and then in ethanol until the required hydrophilicity was obtained: To obtain a fully hydrophilic surface, this ultrasonication procedure was carried out for more than overnight. Immediately after washing the electrode with distilled water, the surface tension was measured for the electrode without drying. Electrode reactions of myoglobins were measured by cyclic voltammetry at 25 °C in a Britton-Robinson (B&R) buffer solution of pH 6.5 using a Toho-Giken 2020/2130 potentiostat with a function generator under nitrogen atmosphere. The area of ca. 5 x 5 mm of the cleaned In_2O_3 electrode was used for electrochemical measurements, and the active area of the working electrode was estimated by using the well-known redox behavior of ferri-/ferro-cyanide in a weakly acidic solution. The platinum plate and Ag/AgCl (sat. KCl) were used as the counter and reference electrodes, respectively. Horse heart and sperm whale skeletal muscle ferrimyoglobins were obtained from Sigma, and was further purified by chromatography using a Whatman CM-52 column.[3] The purity of myoglobin obtained was evaluated[3] by UV-Vis spectroscopy and by using SDS-PAGE technique. The concentration of myoglobin were determined spectroscopically for ferromyoglobin in a phosphate buffer solution (5 mM NaH_2PO_4 + 6.6 mM Na_2HPO_4, pH=7; ionic strength μ=ca. 0.025 M) using the molar absorptivity of 1.14 x 10^5 M^{-1} cm^{-1} at 434 nm.[8]

Figure 1 shows the typical cyclic voltammograms of myoglobins from horse and sperm whale at different hydrophilic surfaces of In_2O_3 electrodes. As the electrode surface becomes more hydrophilic (or larger values of surface tension), better-defined redox waves of both horse and sperm whale myoglobins are clearly seen. Without cleaning the electrode, the In_2O_3 electrode surface is hydrophobic (surface tension of <ca. 30 dyn cm^{-1}) due to adsorbed organic molecules, and no redox wave of myoglobin was observed. However, after removal of adsorbed species, the surface of an In_2O_3 electrode must be rather hydrophilic, because of dissociated surface hydroxide groups. Thus, a usual ultrasonic cleaning in an aqueous cleaning solution gives the value of surface tension of ca. 50 dyn cm^{-1}, but at a such "clean" surface is not enough to obtain well-developed voltammograms of myoglobin. It should be noted that the values of surface tension of the electrode close to that of pure water (72 dyn cm^{-1} at 25 °C) are required to obtain well-defined redox waves. In other words, at the highly hydrophilic surface of In_2O_3 electrode, very clear and stable voltammograms of myoglobin are always obtained, and this is the first clear redox wave reported for sperm whale myoglobin.

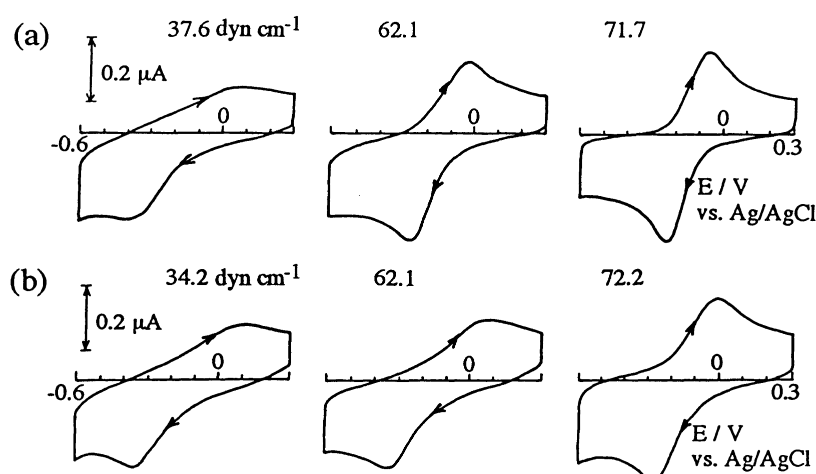

Fig. 1. Cyclic voltammograms of 50 μM myoglobins from (a) horse heart and (b) sperm whale skeletal muscle at In_2O_3 electrodes with different hydrophilic surfaces in a B&R buffer solution (pH 6.5) under nitrogen atmosphere. The value of surface tension of the electrode used is given on each voltammogram. Scan rate: 20 mV/s.

From the voltammograms, the formal redox potential ($E^{o\prime}$), as a midpoint of anodic and cathodic peak potentials, and the diffusion coefficient (D), calculated from the cathodic peak current using a digital simulation technique, were ca. -145 ± 3 mV (vs. Ag/AgCl) and 1.1 x 10^{-6} cm^2 s^{-1}, respectively, which are almost independent of origin of myoglobin (horse or sperm whale) and in good agreement with those reported in the literatures[9] ($E^{o\prime}$=-151 mV vs. Ag/AgCl and D =1.14 x10^{-6} cm^2 s^{-1} for sperm whale myoglobin). No significant difference was observed for these values by changing the type of the buffer solution.

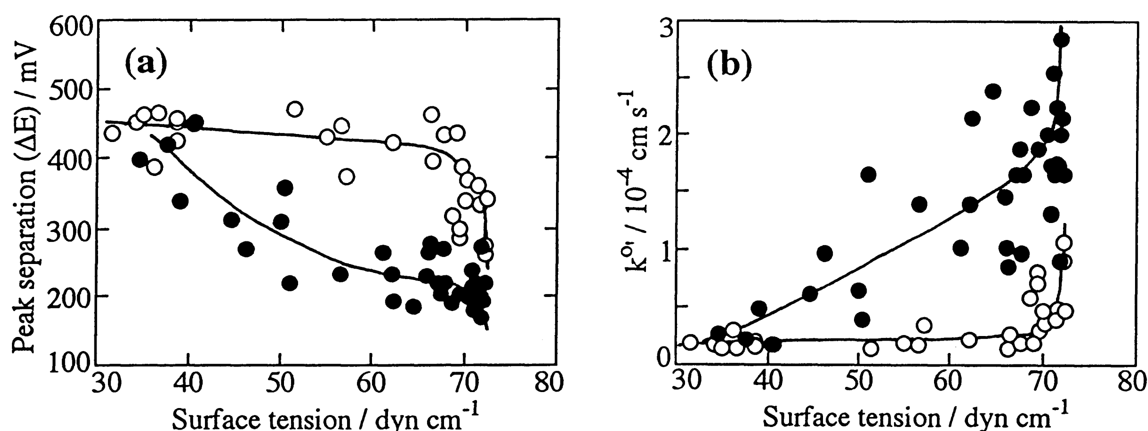

Fig. 2. Change in (a) the peak separation (ΔE) of the voltammogram at a scan rate of 20 mV/s and (b) the formal heterogeneous electron transfer rate constant ($k^{o\prime}$) for 50 μM myoglobin from either horse heart (•) or sperm whale skeletal muscle (o) in a B&R buffer solution (pH 6.5) as a function of the surface hydrophilicity given as the surface tension of the In_2O_3 electrode. Each point indicates the result of an independent electrode.

Figure 2 shows the relationship between the hydrophilicity of the In_2O_3 electrode surface and the kinetics of the electrode reaction of myoglobin given as the peak separation (ΔE) of the observed redox wave (Fig. 2a) and the $k^{o\prime}$ value obtained by using a digital simulation technique for the voltammogram[3] (Fig. 2b). Again, the more the surface becomes hydrophilic, the better the kinetics of myoglobin is shown, although the data are still somewhat scattered. To evaluate the surface hydrophilicity, the contact angle measurement by the droplet method was also carried out using a FACE CA-D contact angle meter (Kyowa Interface Science Co. Ltd.), and similar results to that of Fig. 2 were obtained, but, because this method requires dry electrodes, the contamination of the surface occurred during a drying procedure made difficult to obtain a fully hydrophilic surface. From Fig. 2, the heme edge of myoglobin would approach preferably to the hydrophilic surface of In_2O_3 to give the rapid electron transfer. Furthermore, interestingly, myoglobin from sperm whale seriously requires the very hydrophilic surface for its well-defined electrode reaction, while horse myoglobin begins to give quasi-reversible responses at less hydrophilic surfaces. Also, the largest $k^{o\prime}$ value obtained for horse myoglobin is clearly larger than that for sperm whale myoglobin. The implications of these results are not fully understood at present, but the observed differences may be partly explained in terms of the larger flexibility in structure for horse myoglobin than sperm whale: There are 18 differences in the amino acid sequence between myoglobins from sperm whale skeletal muscle and horse heart, which gives the different isoelectric points[10] (6.8 and 8.25for horse and sperm whale, respectively). However, the differences of amino acid residues mainly appear at the opposite side of the heme edge, resulting in no significant influence in electrochemical properties between these myoglobins, except the $k^{o\prime}$ value. When the living environments of these animals are taken into account, it seems to be reasonable that the myoglobin molecule of sperm whale has a more rigid

structure to be functional even under high pressures. It is also a rather common experience that sperm whale myoglobin is more stable from denaturation during storage or chemical modification of amino acid residues than horse heart myoglobin, suggesting a more solid structure for sperm whale myoglobin. This structural flexibility of horse myoglobin would shorten the path length for electron transfer between the heme and the electrode, and/or increase in the rate of structural change, if any, due to, for example, the change in the spin-state of heme iron during electron transfer, resulting in larger $k^{o\prime}$ values for horse myoglobin. Thus, the dependence of $k^{o\prime}$ value on the origin of myoglobin is interesting and a subject of further investigations from a view point of their biological functions for the adaptability to living environments through dioxygen storage and transport.

The present results would be one of the most important steps for developing protein electrochemistry, and now, by using an In_2O_3 electrode, conventional electrochemical techniques are ready to use for various studies on myoglobin. Also, on the basis of the present study, modified electrodes for myoglobin electrochemistry may be designed, and some attempts are now under way.

Partial financial support of this work by Grant-in-Aid for Scientific Research (No. 04453082) and those on Priority Areas (Nos. 05235102 & 05209225) from the Ministry of Education, Science and Culture, Japan, is gratefully acknowledged.

References

1) For example, F. A. Armstrong, H. A. O. Hill, and N. J. Walton, *Acc. Chem. Res.*, **21**, 407 (1988); I. Taniguchi, "Charge and Field Effects in Biosystems-II," ed by M. J. Allen, S. F. Cleary, and F. M. Hawkridge, Plenum Press, New York (1989), p.91; A. M. Bond and H. A. O. Hill, "Metal Ions in Biological Systems," ed by Helmut Sigel and Astrid Sigel, Marcel Dekker, New York (1991), Vol. 27, Chap. 12, p. 431; M. Tominaga, K. Hayashi, and I. Taniguchi, *Anal. Sci.*, **8**, 829 (1992).
2) B. C. King, F. M. Hawkridge, and B. M. Hoffman, *J. Am. Chem. Soc.*, **114**, 10603 (1992).
3) I. Taniguchi, K. Watanabe, M. Tominaga, and F. M. Hawkridge, *J. Electroanal. Chem.*, **333**, 331 (1992).
4) E. F. Bowden, F. M. Hawkridge, J. F. Chelebowski, E. E. Bancroft, C. Thorpe, and H. N. Blount, *J. Am. Chem. Soc.*, **104**, 746 (1982).
5) E. F. Bowden, F. M. Hawkridge, and H. N. Blount, *J. Electroanal. Chem.*, **161**, 355 (1984); D. E. Reed and F. M. Hawkridge, *Anal. Chem.*, **59**, 2334 (1987).
6) I. Taniguchi, H. Kurihara, K. Yoshida, M. Tominaga, and F. M. Hawkridge, *Denki Kagaku*, **61**, 1043 (1992), and references cited therein.
7) T. Smith, *J. Colloid Interface Sci.*, **75**, 51 (1980), and references cited therein.
8) K. D. Hardman, E. H. Eylar, D. K. Ray, L. J. Banaszak, and F. R. N. Gurd, *J. Biol. Chem.*, **241**, 432 (1966).
9) W. R. Heineman, M. L. Meckstroth, B. J. Norris, and C. Su, *Bioelectrochem. Bioenerg.*, **6**, 577 (1979); J. F. Taylor, *J. Biol. Chem.*, **144**, 7 (1944); M. J. Crumpton and A. Polson, *J. Mol. Biol.*, **11**, 722 (1965).
10) K. D. Hapner, R. A. Bradshaw, C. R. Hartzell and F. R. N. Gurd, *J. Biol. Chem.*, **243**, 683 (1968); A. Bellelli, G. Antonini, M. Brunori, B. A. Springer, and S. G. Silgar, *ibid.*, **265**, 18898 (1990).

(Received July 28, 1993)